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Microfungi associated with withering willow wood in ground contact near Syowa Station, East Antarctica for 40 years

Dai Hirose • Yukiko Tanabe • Masaki Uchida • Sakae Kudoh • Takashi Osono

D. Hirose
College of Pharmacy, Nihon University, Chiba 274-8555 Japan

Y. Tanabe
Graduate School of Frontier Sciences, The University of Tokyo, Chiba 277-8563 Japan

Y. Tanabe • M. Uchida • S. Kudoh
National Institute of Polar Research, Tokyo 173-8515 Japan

17

18 T. Osono (✉)

19 Center for Ecological Research, Kyoto University, Shiga 520-2113 Japan

20 e-mail: tosono@ecology.kyoto-u.ac.jp

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22 **Abstract**Data are rather lacking on the diversity of microfungi associated with
23 exotic plant substrates transported to continental Antarctica. We examined the
24 diversity and species composition of microfungi associated with withering woody
25 shoots of saplings of *Salix* spp. (willows) transplanted and in ground contact
26 near Syowa Station, East Antarctica for more than 40 years. The willow
27 saplings originated from Hokkaido, Northern Japan, and were experimentally
28 transplanted in 1967-1968, but died within a few years. Dead willow shoots,
29 unbranched and standing on bare ground for approximately 50 years, were used
30 for the isolation of fungi with the surface disinfection method. A total of 43
31 isolates were retrieved from 32 (78%) of the 41 shoots tested. The fungal isolates
32 were classified into 18 molecular operational taxonomic units (MOTUs) based on

the similarity of rDNA ITS sequences at the 97% criterion. Leotiomycetes was the most common class in terms of the number of isolates and MOTUs, followed by Dothidiomycetes, Sordariomycetes, and Eurotiomycetes. Molecular phylogenetic affinities suggested that the closest relatives of the MOTUs were saprobic and root-associated fungi. The result of the present study suggested that *Cadophora luteo-olivacea* is widespread in soils throughout Antarctica and likely indigenous.

Keywords Continental Antarctica • Fungi • Root endophyte • *Salix* • Syowa Station

Introduction

Ice-free regions of continental Antarctica, comprising only about 2% of the continent, are cold and arid, and strong selection pressures are imposed on plant establishment and soil development. Despite the harsh environment, previous

studies have reported the occurrence of free-living fungi in soils and in association with bryophytes in coastal outcrops of continental Antarctica (e.g. Azmi and Seppelt 1997; Tosi et al. 2002, 2005; Newsham et al. 2009). Recent studies have examined fungal populations in historically-introduced exotic materials and found a significant overlap of fungi isolated from these materials and fungi isolated from environmental samples in pristine locations (Farrell et al. 2011). A significant effect of exotic substrates on indigenous soil fungi has also been found (Arenz et al. 2011). However, data are still lacking regarding the diversity of microfungi associated with exotic plant substrates transported to continental Antarctica. The purpose of the present study is to examine microfungi associated with withering woody shoots of saplings of *Salix* spp. (willows) in ground contact in Syowa Station, East Antarctica for 40 years.

Materials and methods

Study site and sample collection

65

66 Samples were collected near Syowa Station on East Ongul Island, Lützow-Holm
67 Bay, East Antarctica (60°00'47"S, 39°34'57"E, 16 m a.s.l.). In February 1967,
68 saplings of dwarf deciduous shrubs *Salix pauciflora* and *S. reinii*, 10-20 cm in
69 height and originating from Hokkaido, Northern Japan, were transplanted at
70 experimental sites near Syowa Station by Dr. T. Hoshiai of the 8th Japanese
71 Antarctic Research Expedition (JARE-8) to test their growth and survivorship.
72 These saplings endured through winter, sprouted, and bloomed in the next
73 summer of 1968, but not all sprouted in the summer of 1969 (Hoshiai 1970).
74 Additional saplings were transplanted by Dr. Y. Endo of JARE-9 in 1968, giving
75 a similar result of the sapling producing leaves the next year but dying within a
76 few years because of the adverse environment of Antarctica (Hoshiai 1970).
77 During JARE-51 in 2009-2010, we found dead willow shoots still standing on the
78 experimental site. In February 2010, a total of 41 withering shoots (aboveground
79 parts without leaves, soil, or belowground parts, approximately 3 cm in height,
80 and 1-3 mm in basal diameter) were collected with tweezers, preserved in paper

bags, stored at 2°C, and taken back to the laboratory in Japan.

Fungal isolation

Fungi were isolated from shoots using the surface disinfection method according to Osono et al. (2012). The surface-disinfected shoots were plated on 9-cm Petri dishes containing 2% lignocellulose agar (LCA) modified as described by Miura and Kudo (1970) (glucose 0.1%, KH_2PO_4 0.1%, $MgSO_4 \bullet 7H_20$ 0.02%, KCl 0.02%, $NaNO_3$ 0.2%, yeast extract 0.02%, and agar 2% (w/v)), two shoots per plate. Note that the modified LCA of Miura and Kudo (1970) does not contain lignin or other recalcitrant compounds. The modified LCA was used because its low glucose content suppresses the overgrowth of fast-growing fungal species (Osono and Takeda 1999). The plates were incubated in darkness at 10°C and observed for 4 weeks after the disinfection. Any fungal hyphae or spores appearing on the plates were subcultured onto fresh LCA plates, incubated, and observed micromorphologically. Isolates were then used for molecular analysis as

97 described below.

98

99 Molecular methods

100

101 Genomic DNA was extracted from mycelia that had been cultured on 2.5% malt
102 extract agar overlaid with a cellophane membrane following the modified CTAB
103 method described by Matsuda and Hijii (1999). Polymerase chain reactions
104 (PCR) were performed using a Quick Taq HS DyeMix (Toyobo, Osaka, Japan).
105 Each PCR reaction contained a 50 µl mixture (21 µl distilled water, 25 µl master
106 mix, 3 µl ca. 0.5ng/µl template DNA, and 0.5 µl of each primer (final, 0.25 µM)).
107 To PCR amplify the region including the rDNA ITS and 28S rDNA D1-D2
108 domain, the primer pair ITS1f (Gardes and Bruns 1993) and LR3 (Vilgalys and
109 Hester 1990) was used. Each DNA fragment was amplified using a PCR thermal
110 cycler (DNA engine; Bio-Rad, Hercules, CA, USA) using the following thermal
111 cycling schedule. The first cycle consisted of 5 min at 94°C, followed by 35 cycles
112 of 30 s at 94°C, 30 s at 50°C for annealing, 1 min at 72°C, and a final cycle of 10

113 min at 72ºC. The reaction mixture was then cooled at 4ºC for 5 min. PCR
114 products were purified with a QiAquick PCR Purification Kit (Qiagen,
115 Germany) according to the manufacturer's instructions.

116 Purified PCR products were sequenced by FASMAC Co., Ltd.
117 (Kanagawa, Japan). Sequencing reactions were performed in a Gene Amp PCR
118 System 9700 (Applied Biosystems, USA) using a BigDye Terminator V3.1
119 (Applied Biosystems), following the protocols supplied by the manufacturer. The
120 fluorescent-labeled fragments were purified from the unincorporated
121 terminators using an ethanol precipitation protocol. The samples were
122 resuspended in formamide and subjected to electrophoresis in an ABI 3730xl
123 sequencer (Applied Biosystems).

124 The sequences determined in this study were deposited in the DNA
125 Data Bank of Japan (DDBJ) (AB752244-AB752287). The rDNA ITS sequences
126 were compared with available rDNA sequences in the GenBank database using
127 BLASTN searches (Altschul et al. 1990). For phylogenetic analysis, MAFFT ver.
128 6 (Katoh and Toh 2008) was used for preliminary multiple alignments of

nucleotide sequences. Final alignments were manually adjusted using BioEdit (Hall 1999). Alignment gaps were treated as missing data, and ambiguous positions were excluded from the analysis. The phylogenetic tree was conducted by maximum likelihood (ML) methods (Felsenstein 1981) with the best fit nucleotide substitution model based on the lowest Bayesian Information Criterion (BIC) score. To estimate clade support, the bootstrap procedure of Felsenstein (1985) was employed with 1000 replicates. These analyses were carried out using MEGA5 (Tamura et al. 2011).

The isolates were grouped into molecular operational taxonomic units (MOTUs) according to the similarity of rDNA ITS sequences at the 97% criterion. The frequency of occurrence of MOTU was calculated as a percentage of the number of shoots from which a MOTU was detected compared with the total number of shoots tested (i.e. 41).

Results

Fungi were isolated from 32 (78%) of the 41 shoots tested for isolation. A total of 43 isolates were obtained, and these were classified into 18 MOTUs (Table 1, Fig. 1). Leotiomycetes was the most frequent class, including 29 isolates of 10 MOTUs, followed by Dothidiomycetes (9 isolates, 4 MOTUs), Sordariomycetes (3 isolates, 2 MOTUs), and Eurotiomycetes (2 isolates, 2 MOTUs) (Fig. 2). The most frequent MOTUs were MOTU1 in the Leotiomycetes that had 100% sequence match of the ITS region to *Cadophora luteo-olivacea* (7 isolates), MOTU9 in Leotiomycetes (7 isolates), and MOTU18 in Dothidiomycetes (5 isolates) (Table 1, Fig. 2).

Discussion

Some of the microfungi associated with dead willow shoots in the present study are classed as saprobic fungi (Table 1). For example, *Cadophora luteo-olivacea* (MOTU1) is a saprobe occurring in many habitats including wood, soil, and plants (Gramaje et al. 2011). Several *Cadophora* species, including *C.*

161 *luteo-olivacea*, have also been isolated from soils and historic wood along the
162 Ross Sea region of Antarctica (Arenz et al. 2006) and have the potential to cause
163 soft rot in wood (Blanchette et al. 2004). Similarly, *Phialocephala lagerbergii,*
164 which had 99% sequence match of the ITS region to MOTU3, is known to be a
165 wood-inhabiting fungus (Grünig et al. 2009). *Geomyces vinaceus*, an anamorph
166 of *Pseudogymnoascus roseus* and which had 100% sequence match of the ITS
167 region to MOTU8, is associated with wood, soil, and roots (Rice and Currah
168 2006). *Coniochaeta lignaria*, which had 99% sequence match of the ITS region to
169 MOTU13, has been shown to have lignocellulose-degrading enzymes (Lopez et al.
170 2007), which can facilitate growth and energy acquisition in dead willow shoots
171 consisting of structural lignin and cellulose polymers.

172 We noted that root-associated microfungi were isolated frequently from
173 the dead willow shoots (Table 1). For example, *Ilyonectria robusta* and *Phoma*
174 *sclerotioides*, which had 99% sequence match of the ITS region to MOTU14 and
175 MOTU17, respectively, are root-rot fungi (Wunsch and Bergstrom 2011; Cabral
176 et al. 2012). *Phialocephala fortinii*, which had 99% sequence match of the ITS

177 region to MOTU5, and also possibly MOTU2 in *Phialocephala*, is a common
178 endophyte of plant roots and is widespread in sub-Antarctic ecosystems and also
179 present in continental Antarctica (Grünig et al. 2008; Newsham et al. 2009).
180 Jumpponen et al. (2003) detected a DNA sequence with 99% similarity to *P.*
181 *fortinii* in a rhizoid of the liverwort *Cephaloziella varians* on the Antarctic
182 Peninsula.

183 It is unclear whether these fungi were widespread or localized in their
184 distribution in Antarctica and whether they were indigenous to Antarctica or
185 introduced along with the saplings in soil from Japan. MOTU1, one of the most
186 frequent taxa (Table 1), had 99% to 100% sequence match (with query coverage
187 between 89% and 97%) of the ITS region to *Cadophora luteo-olivacea* isolated
188 from wood and soil in the Ross Sea Region (DQ317327, Arenz et al. 2006;
189 GU212374, Blanchette et al. 2010) and along the Antarctic Peninsula (FJ911899,
190 Rosa et al. 2010; HQ438025, Gonçalves et al. 2012). This result suggested that
191 this fungus is widespread in soils throughout Antarctica and likely indigenous.
192 Similarly, *Geomyces vinaceus* (OTU8) was isolated from moss samples in

Victoria Land on the west coast of the Ross Sea (Tosi et al. 2002), but the distribution of this fungus in Antarctica remains unknown and deserve further researches.

It is unclear whether the fungi isolated in the present study were active or dormant in dead shoots. However, the supply of exotic woody substrates, such as dead willow shoots, can contribute to fungal abundance, as the natural lack of organic material in Antarctica limits the densities of fungal populations (Arenz et al. 2011). To exist in Antarctica, fungi need to be able to tolerate the harsh environment, and Antarctic fungi have a variety of physiological traits that enable them to survive under cold and dry conditions (Robinson 2001), including cold tolerance, accumulation of intercellular trehalose and polyols, secretion of antifreeze proteins, and enzymes active at low temperatures. Future studies will include physiological evaluations of these fungal isolates and measurements of activity at low temperatures.

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Hirose et al. Table 1.

Table 1 The number of isolates and blast identity results (in percentage) of fungal molecular operational taxonomic units (MOTUs) isolated from withering willow shoots and sequence accession number for the closest relative found at GenBank.

Class	MOTU	Number of isolates	Closest match at Genbank (Accession number)	Sequence similarity %
Leotiomycetes	1	7	*Cadophora luteo-olivacea* (GU128589)	100
	9	7	Leotiomycetes sp. (JQ759481)	99
	2	4	*Phialocephala* sp. (FM999988)	99
	7	3	Leotiomycetes sp. (JQ758759)	99
	5	2	*Phialocephala fortinii* (EU888625)	99
	8	2	*Geomyces vinaceus* (AJ608972)	100
	3	1	*Phialocephala lagerbergii* (AB190400)	99
	4	1	Helotiales sp. (AB598096)	92
	6	1	*Clathrosporium intricatum* (EF029192)	95
	10	1	*Tetracladium* sp. (AJ890435)	99
Eurotiomycetes	11	1	*Exophiala salmonis* (GU586858)	99
	12	1	*Penicillium turbatum* (AY213679)	100
Sordariomycetes	14	2	*Ilyonectria robusta* (JF735265)	99

	13	1	*Coniochaeta ligniaria* (AY198390)	99
Dothidiomycetes	18	5	Dothideomycetes sp. (JQ759636)	98
	15	2	Leptosphaeria sp. (GU934537)	99
	16	1	*Phoma* sp. (HM589351)	100
	17	1	*Phoma sclerotioides* (FJ179158)	99

1

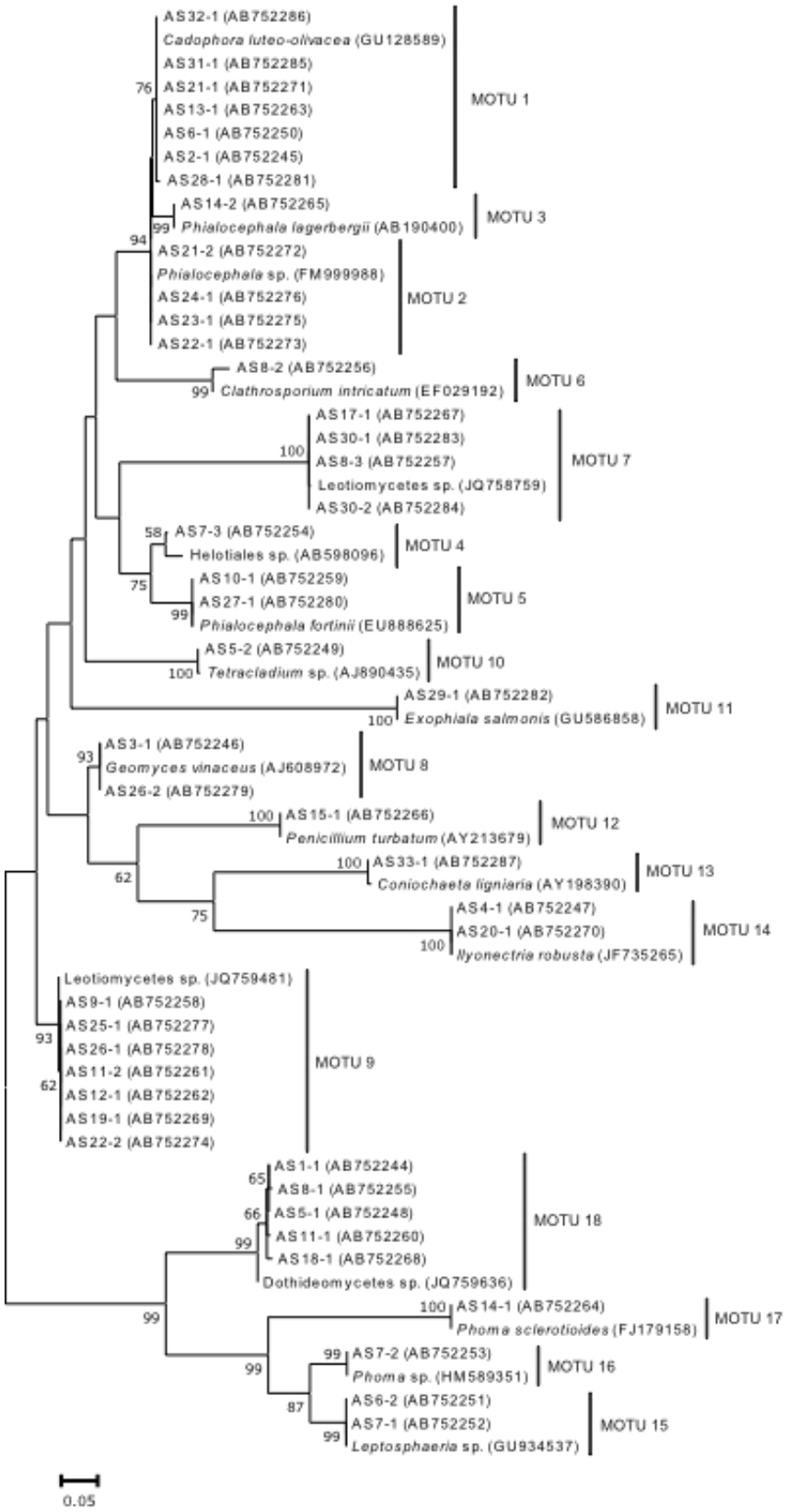
Figure legend

Fig. 1 Maximum-likelihood (ML) phylogeny inferred from rDNA ITS sequences including 18 fungal molecular taxonomic units (MOTUs) isolated from withering willow shoots. The evolutionary model used was the Kimura 2-parameter model (Kimura 1980) with a discrete Gamma distribution (+G, parameter = 0.7952) and a proportion of Invariant sites (+I, 34.3127% sites) to allow for non-uniformity of rates among sites. Bootstrap values for the ML analysis are indicated for corresponding branches.

Fig. 2 Rank-abundance relationship of fungal molecular taxonomic units (MOTUs) isolated from withering willow shoots. Black bar, Leotiomycetes; blank bar, Eurotiomycetes; shaded bar, Sordariomycetes; gray bar, Dothidiomycetes.

1 Hirose et al. Fig. 1

2



1 Hirose et al. Fig. 2